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Heterologous expression of the naphthocyclinone hydroxylase gene from *Streptomyces arenae* for production of novel hybrid polyketides

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Abstract

Streptomyces arenae produces at least four different isochromanequinone antibiotics, the naphthocyclinones, of which the β- and γ-form are active against Gram-positive bacteria. The naphthocyclinone biosynthesis gene cluster was isolated from *Streptomyces arenae* DSM 40737 and by sequence analysis the minimal polyketide synthase genes and several genes encoding tailoring enzymes were identified. Southern blot analysis of the naphthocyclinone gene cluster indicated that a 3.5 kb *Bam*HI fragment located approximately 9 kb downstream of the minimal PKS genes hybridizes to the *schC* hydroxylase DNA probe isolated from *S. halstedii*. Two complete and one incomplete open reading frames were identified on this fragment. Sequence analysis revealed strong homology to the genes of the *act*VA region of *S. coelicolor*, to several (suggested) hydroxylases and a putative FMN-dependent monooxygenase. The proposed hydroxylase, encoded by *ncnH*, could hydroxylate aloesaponarin II, a molecule that is produced by the actinorhodin minimal polyketide synthase in combination with the actinorhodin ketoreductase, aromatase and cyclase. Furthermore, this enzyme is capable of accepting additional polyketide core structures that contain a 5-hydroxy-1,4-naphthoquinone moiety as substrates which makes it an interesting tailoring enzyme for the modification of polyketide structures.

Abbreviations: aa – amino acid(s); *act* – actinorhodin biosynthetic genes; bp – base pair(s); COSY – correlation spectroscopy; 3,8 DMAC – 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid; DNA – deoxyribonucleic acid; DSM – Deutsche Sammlung von Mikroorganismen; *fren* – frenolicin biosynthetic genes; HMBC – heteronuclear multiple-bond correlation; HMQC – heteronuclear multiple-quantum correlation; HPLC – high performance liquid chromatography; kb – kilobase pair(s); MS – mass spectroscopy; *ncn* – naphthocyclinone biosynthetic genes; NMR – nuclear magnetic resonance; nt – nucleotide(s); ORF – open reading frame; RBS – ribosome binding site; *tcm* – tetracenomycin biosynthetic genes

Introduction

Polyketides are structurally and chemically diverse secondary metabolites which exhibit a variety of important biological functions including antibiotic, antitumor or immunosuppressive activities. Most of the known polyketides are synthesized by streptomycetes. Polyketides provide pharmaceuticals for almost every important therapeutic area. There are only about 10,000 polyketides known today from which an extraordinarily large number of pharmaceutical products have been derived. Biosynthesis of those complex molecules depends on the action of so-called tailoring enzymes which decorate the polyketide backbone produced by the minimal PKS to yield the final chemical structure. Tailoring enzymes can be glycosyltransferases, methyltransferases, decarboxylases, hydroxylases and others.

Hydroxylation reactions are quite common in the biosynthesis of polyketides many of which are clin-

ically important molecules. For example, the two therapeutically relevant polyketides, doxorubicin and erythromycin, both are hydroxylated molecules. The antitumor anthracycline antibiotics daunorubicin and doxorubicin produced by *Streptomyces peucetius* are hydroxylated in two reactions (Lomovskaya et al. 1999). The first hydroxylation, at position 11 of aklavinone, yields ϵ-rhodomycinone. Although the exact biological role of 11-hydroxylation of aklavinone is not yet clear, it is supposed to increase the toxicity of the compound (Oki 1988). The second hydroxylation converts daunorubicin to doxorubicin by C-14 hydroxylation.

Biosynthesis of erythromycin A involves three hydroxylation steps by two different P_{450}-monooxygenases (EryF and EryK). These enzymes catalyze the conversion of 6-deoxyerythronolide-B to erythronolide-B (EryF) and the hydroxylation of erythromycin D to erythromycin C and erythromycin B to erythromycin A, respectively (EryK) (Weber et al. 1991; Stassi et al. 1993). In addition, many other polyketide core molecules undergo hydroxylation reactions during biosynthesis (e.g. tetracenomycin, tetracycline, urdamycin and others).

In recent years numerous examples have shown that the combination of genes from different bacterial polyketide clusters can lead to the production of novel recombinant polyketides (for a review see Hopwood 1997). In order to enlarge the chemical diversity of recombinant polyketides we were interested in isolating new tailoring enzymes which can modify unnatural substrates. One of our goals was to identify novel hydroxylases which could be used for production of new active hybrid polyketide molecules. *S. arenae* produces naphthocyclinones, isochromanequinone antibiotics which exhibit activity against Gram-positive bacteria. From the chemical structure of naphthocyclinone it could be concluded that there is at least one hydroxylase involved in naphthocyclinone biosynthesis. Here we report the cloning, characterization and expression of the naphthocyclinone hydroxylase gene from *Streptomyces arenae* DSM40737.

Material and methods

Bacterial strains and culture conditions

All *E. coli* and *Streptomyces* strains used in this work are listed in Table 1. *E. coli* was grown at 37 °C either in dYT liquid medium or on dYT agar plates (Sambrook et al. 1989), both supplemented with the appropriate antibiotics for plasmid selection as required. *Streptomyces coelicolor* CH999 (McDaniel et al. 1993) the host for expression of polyketide genes was grown at 30 °C in TSB liquid medium or R5 agar plates (Hopwood et al. 1985). If required thiostrepton was added to 12.5 mg/l.

Plasmids

Plasmids used or constructed during this work with their relevant markers are listed in Table 1. The plasmid pETR351 was constructed by insertion of a 3.5 kb *Bam*HI fragment from pKMA which hybridizes with *schC* into the vector pJOE2114.1. The proposed hydroxylase gene, *ncnH*, was isolated from pETR351 as an *Eco*RI fragment and ligated into the *Eco*RI site of the vectors pSEK33, pRM5, pETR511 and pSEK26 resulting in the plasmids pETR509, pETR512, pETR515 and pETR537, respectively.

DNA isolation, manipulation and transformation of bacterial cells

For plasmid preparation from *E. coli* either a method described by Lee & Rashed (1990) or a QIAprep spin Kit (Qiagen, Germany) was used. Isolation of plasmid DNA from *Streptomyces* was performed according a protocol described by Hopwood et al. (1985). All standard DNA techniques and Southern blot analysis were done as described by Sambrook et al. (1989). Restriction endonucleases, modifying enzymes and T4 DNA ligase were obtained from commercial sources and used according to the manufacturer's recommendations. DNA probes were digoxigenin-labeled using the Random Primed DNA Labeling Kit (Roche, Switzerland). Transposon mutagenesis of the plasmid pETR351 for the generation of sequencing templates was done as described by Fischer et al. (1996). The method of Mandel & Higa (1979) was used for preparation and transformation of competent *E. coli* cells. Protoplast formation and PEG-mediated transformation of *Streptomyces* was performed according to a protocol described elsewhere (Hopwood et al. 1985). To obtain non-methylated DNA for transformation of *S. coelicolor* CH999, plasmids were isolated from *E. coli* ET12567.

DNA sequencing and analysis

Templates for sequencing of double stranded DNA were either generated by subcloning of DNA frag-

Table 1. Bacterial strains and plasmids used in this work

Bacterial strain/ plasmid	Description	Reference/source
E. coli XL 1-Blue	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [F′ *proAB lacI*^{q} *Z*ΔM15 Tn *10* (Tet^R)]	Bullock et al. 1987
E. coli ET12567	*supE*44 *hsdS*20 ($r^-_B m^-_B$) *ara*-14 *proA*2 *lacY galK*2 *rpsL*20 *xyl*-5 *mtl*-1 *dam*$^-$ *dcm*$^-$ *hsdM*$^-$ Cm^R	MacNeil et al. 1992
E. coli JM108 F′ (::Tn*5491*)	*recA*1 *endA*1 *gyrA*96 *hsdR*17 *supE*44 *relA*1 *thi* Δ (*lac* – *proAB*) F′ (::Tn5491 *proA*$^+$*B*$^+$ *lacI* Tc^R)	Fischer et al. 1996
E. coli HB101	*supE*44 *hsdS*20 (r^-_B m^-_B) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	Boyer & Roulland-Dussoix 1969
S. arenae DSM 40737	Wild-type	Pridham et al. 1958
S. coelicolor CH999	*S. coelicolor* A3(2) derivative, Δ*act*, *redD*60, *proA*1, *argA*1, SCP1$^-$, SCP2$^-$	McDaniel et al. 1993
pIC19H	*lacZα*	Marsh et al. 1984
pKMA	33 kb *S. arenae* chromosomal DNA fragment in pKC505	Brünker et al. 1999
pETR351	3.5 kb *Bam*HI fragment from pKMA (hybridizing to *schC*) in pJOE2114	this work
pETR509	*tcm* PKS, *ncnH*	this work
pETR511	*act* KR, *tcm* PKS	this work
pETR512	*act* KR, PKS, ARO, CYC, *ncnH*	this work
pETR515	*act* KR, *tcm* PKS, *ncnH*	this work
pETR537	*act* KR, *fren* PKS, *fren* ARO, *act* CYC, *ncnH*	this work
pJOE2114	*mel*, *res*	Fischer et al. 1996
pRM5	*act* KR, PKS, ARO, CYC	McDaniel et al. 1993
pSEK26	*act* KR, *fren* PKS, *fren* ARO, *act* CYC	McDaniel et al. 1995
pSEK33	*tcm PKS*	McDaniel et al. 1994

act: actinorhodin biosynthesis genes from *S. coelicolor*.
fren: frenolicin biosynthesis genes from *S. roseofulvus*.
ncn: naphthocyclinone biosynthesis genes from *S. arenae*.
tcm: tetracenomycin biosynthesis genes from *S. glaucescens*.

ments into pIC vectors (Marsh et al. 1984) or by using a transposon based deletion method described by Fischer et al. (1996). Universal forward (5′ AGGGTTTTCCCAGTCACG ACGTT 3′ or 5′ GTAAAACGACGGCCAGT 3′) and reverse (5′ GAGCGGATAACAATTTCACACAGG 3′) primers (IRD40 labeled) purchased from MWG-Biotech (Ebersberg, Germany) and Thermosequenase™ (Amersham Pharmacia Biotech) were used in the dideoxy chain termination sequencing reactions (Sanger et al. 1977). The sequencing reactions were separated and analyzed with an automated LICOR DNA 4000L sequencer (Lincoln, NE, USA) using the BaseImagIR™ V.2.30 software package. The nucleotide sequence was analyzed with programs of the DNAstar software, version 3.15 (1997). Database searches (Genbank) were run with the programs BlastP, BlastN or BlastX (V 2.0) on the blast electronic mail server from the National Center for Biotechnology Information, Bethesda, Maryland, USA.

Extraction, analysis and purification of polyketides

Polyketide producing *Streptomyces* strains were incubated at 30 °C for 20 days on R5 agar plates. The agar was extracted four times with 1 volume of ethylacetate:methanol:acetic acid (79:20:1). For analytical HPLC a C18 reverse phase column (Macherey Nagel, CC250/4 Nucleosil 100) was used. Polyketides were separated with an acetonitrile/H_2O gradient (20–80% including 1% acetic acid) with a constant flow of 0.5 ml/min. Spectra (210–700 nm) were monitored using a diode array detector (Perkin Elmer, Series 200). For purification of a compound the culture extract

was prepurified with a silica gel flash column (Baker analyzed® Reagent, particle size 40 μm, J.T. Baker), dried, dissolved in methanol and separated by preparative HPLC system (Waters DeltaPrep 4000) using a C18 reverse phase column (Delta Pack C18, 300Å, 15 μm, 40 $\times$ 100 mm). During the run the absorbance at 280 nm was monitored, fractions of interest were collected, dried and analyzed by NMR and MS.

Biological activity of the purified compound P-512 was determined by our industrial partner using a protocol that is based on the method described by Roth et al. (1997) using SYTOX Green, a nucleic acid stain to determine cell viabilities. Briefly, upon binding to DNA fluorescence of SYTOX Green is 1000 fold enhanced. SYTOX Green easily penetrates compromised membranes of Gram-positive and Gram-negative bacteria but is completely excluded from living cells. Thus an increase of fluorescence in the test culture is indicative for biological activity of a compound. In the assay overnight cultures of *E. coli* or *B. subtilis* were exposed to purified P-512 (100, 50, 25, 12.25 and 6.25 μg/ml) and SYTOX Green stain. The increase of relative fluorescence in combination with the decrease of cell density (measured as the decrease in OD_{600}) allows conclusions about the antibacterial activity of the tested substance.

Structure elucidation

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, containing 5% CD_3OD, and the solvent signals (of $CHCl_3$ at 7.26 ppm in the ^{1}H NMR spectrum and $CDCl_3$ at 77.0 ppm in the ^{13}C NMR spectrum) were used as reference. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH}$=145 Hz and $^nJ_{CH}$=10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol SX102 spectrometer, while the melting point (uncorrected) was determined with a Reichert microscope.

Results and discussion

Identification, subcloning and sequencing of the naphthocyclinone hydroxylase gene

Recently the isolation and characterization of the naphthocyclinone gene cluster from *Streptomyces arenae* DSM40737 has been reported (Brünker et al. 1999). Southern blot analysis of the cluster using a *schC* specific probe from *S. halstedii* (Blanco et al. 1993) indicated the presence of a putative hydroxylase gene located on a 3.5 kb *Bam*HI fragment downstream of the minimal PKS genes. This *Bam*HI fragment was cloned into the vector pJOE2114 resulting in the plasmid pETR351 which was used as a target for Tn*5491* mediated transposon mutagenesis for generation of sequencing templates. By using the transposon based mutagenesis and subcloning of DNA fragments both strands of the 3.5 kb *Bam*HI fragment which hybridizes to *schC* were sequenced. The nucleotide sequence was deposited at GenBank (accession number AF218066).

Homology comparison with data from GenBank revealed two complete and one incomplete open reading frame on the sequenced 3.5 kb fragment (Figure 1, Table 2). All three open reading frames show the typical *Streptomyces* codon usage and a GC-content between 75% and 77%. The first 572 nucleotides of the sequenced fragment encode the C-terminal part of a protein which is to 65% identical to a deduced peptide sequence from the *S. violaceoruber* granaticin cluster (*gra*-ORF29; Ichinose et al. 1998). The function of this gene is not known, but it resembles the α-subunit of a FMN-dependent monooxygenase from *Xenorhabdus luminescens* (Ichinose et al. 1998). The product of *gra*-ORF29 is speculated to be involved in sugar biosynthesis in granaticin production. Since naphthocyclinone is not glycosylated the function of the *ncn*-ORF3 product remains unclear. Downstream of *ncn*-ORF3 an additional open reading frame (*ncn*-ORF2) is located at position 717 to 1580, encoding a putative protein of 287 aa with a calculated molecular mass of 30.13 kDa. The deduced peptide sequence is to 41% identical to a putative protein (*act*VA-3) of the *act*VA region of the *S. coelicolor* actinorhodin gene cluster. Furthermore the product of *ncn*-ORF2 also shows significant homology to two deduced proteins of the *S. violaceoruber* granaticin gene cluster (*gra*-ORF28 and *gra*-ORF30). The functions of those proteins is so far unknown but *act*VA-3 seems to be involved in C-6 hydroxylation during actinorhodin

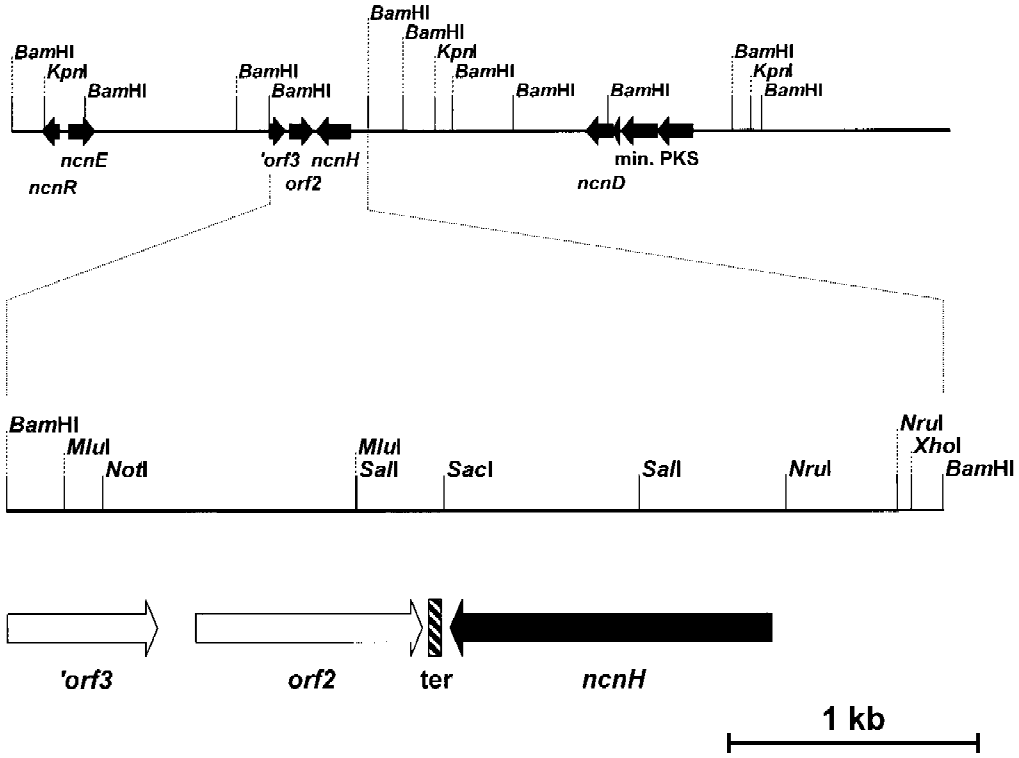

Figure 1. Preliminary physical map of the 33 kb chromosomal region representing the naphthocyclinone cluster from *Streptomyces arenae* DSM40737. The 3.5 kb *Bam*HI fragment hybridizing to the *schC* probe (0.55 kb PCR fragment generated with the primers 5′ GCAGGGGATCCGCCGGGCCG 3′ and 5′ CTCTCGGGTACCAGGGGGCG 3′ with pUO6030 as the template) is enlarged and the identified open reading frames and the putative terminator region (ter) are indicated.

Table 2. Summary of sequence analysis of the 3.5 kb *Bam*HI fragment

Gene	Length [nt/aa]	GC content [%]	Mr *	Putative RBS (distance to start)		Homology [% identity]
'ncn-ORF3	570/189	75.78	–	–		65% *gra*-ORF29
ncn-ORF2	864/287	76.15	30.13	GGAGG	(4)	41% *act*VA-3
						43% *gra*-ORF28
						46% *gra*-ORF30
ncnH	1218 /405	77.00	42.45	GGACGG	(6)	52% *act*VA-5
						50% *gra*-ORF21

*Molecular mass [kDa].

biosynthesis (Caballero et al. 1991). All four proteins are very similar in size (*act*VA-3: 281 aa; *gra*-ORF28:266 aa; *gra*-ORF30: 282 aa; *ncn*-ORF2: 287 aa). A third open reading frame (*ncnH*), 1218 nt in length, starting at position 2904 is located downstream of *ncn*-ORF2. This open reading frame, encoding a protein of 405 aa with a calculated molecular mass of 42.45 kDa is orientated in the opposite direction of *ncn*-ORF2. The deduced peptide sequence of *ncnH* shows a high degree of homology to *act*VA-5 of *S. coelicolor* and *gra*-ORF21 of *S. violaceoruber*, which are both probably involved in hydroxylation reactions in the actinorhodin and granaticin biosynthetic pathway, respectively. While NcnH and the product of *gra*-ORF21 are very similar in size, 405 aa and 400 aa, respectively, the *act*VA-5 gene product is only 381 aa in length. A multiple sequence alignment of all three proteins indicate that *act*VA-5 mainly is shorter at the

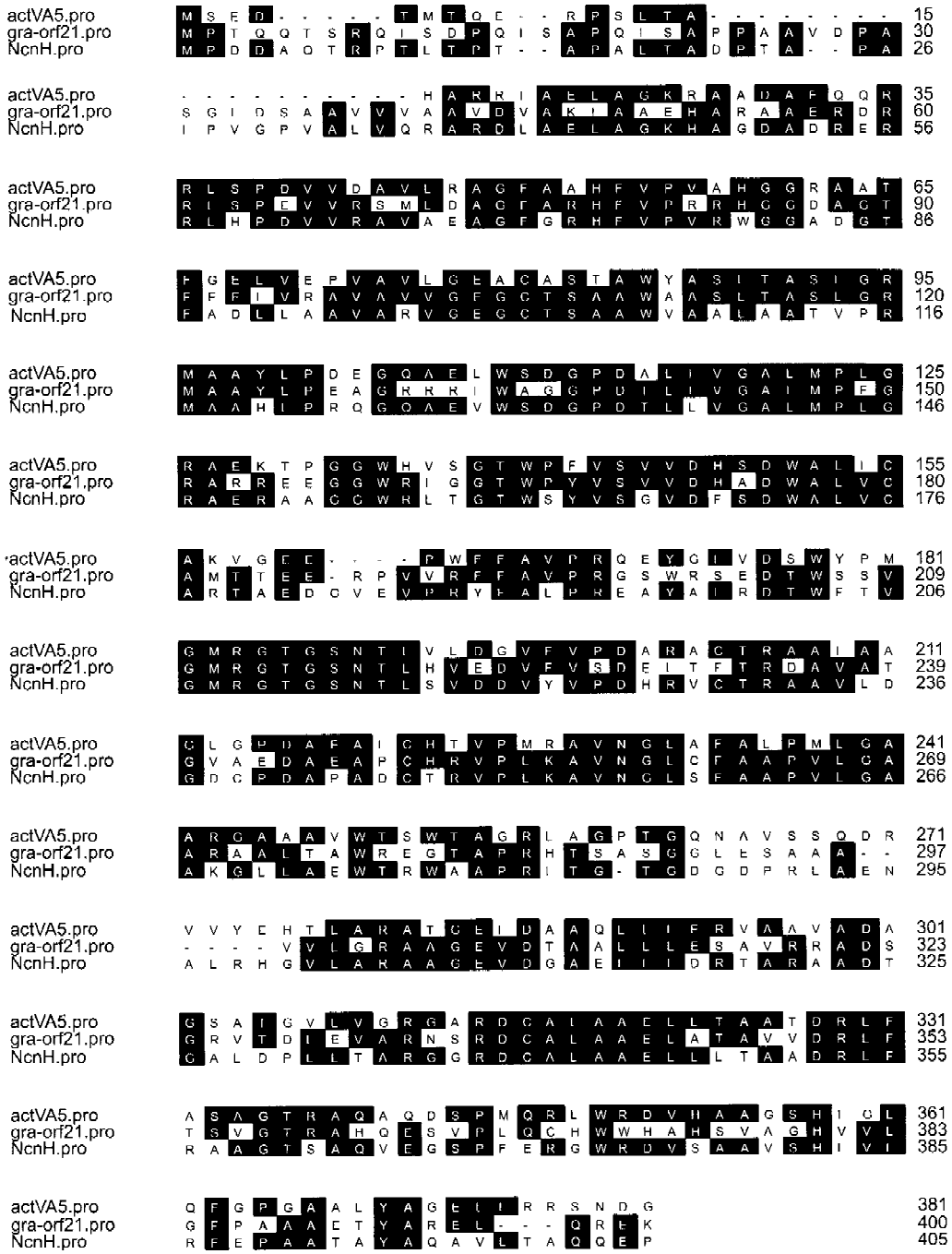

Figure 2. Multiple sequence alignment of the deduced peptide sequences of NcnH, ActVA-5 (Caballero et al. 1991) and Gra-ORF21 (Ichinose et al. 1998). Numbers indicate the positions of the amino acids within the sequence. Highly conserved aa (identical in at least two proteins) are shown in white letters with black background.

N-terminus compared to NcnH and the *gra*-ORF21 peptide (Figure 2). Phylogenetic analysis of six different polyketide hydroxylases indicates that they are divided into two different groups with a common ancestor (data not shown). The hydroxylases of the tetracycline-, tetracenomycin- and *S. halstedii* spore pigment cluster (OtcC, TcmG and SchC, respectively) represent one group, whereas NcnH, Gra-ORF21 and ActVA-5 fall into the second group. These groups cannot only be distinguished by sequence comparison (there is only a low degree of homology between the two groups) but they also are quite different in size. The proteins of the ActVA-5 group consist of approximately 400 aa whereas the peptides belonging to the tetracycline hydroxylase group are about 470–572 aa in length. OtcC, TcmG and SchC all belong to a family of flavin-type bacterial hydroxylases with a well-conserved common N-terminal cofactor binding site (**G**-X-**G**-X-X-**G**; Wierenga et al. 1985). No such obvious cofactor binding site or any other consensus motif could be identified within the protein sequences of NcnH, ActVA-5 or Gra-ORF21. The lack of those consensus motifs implicates that these proteins represent a new class of hydroxylating enzymes which are quite different from the already known hydroxylases.

In the intergenic region between *ncn*-ORF2 and *ncnH* a perfect inverted repeat of 12 nucleotides was identified (not shown) which could form a stable stem-loop structure (where the loop is 15 bp in length) which might function as a transcriptional terminator.

Heterologous expression of ncnH

In order to investigate the function of the proposed naphthocyclinone hydroxylase, the *ncnH* gene was co-expressed in combination with other polyketide core producing genes from different clusters. For that purpose *ncnH* was isolated as an *Eco*RI fragment from the plasmid pETR351 and inserted into the *Eco*RI site of the vectors pRM5, pSEK33, pETR511 and pSEK26 downstream of the PKS genes. After transformation of *S. coelicolor* CH999 with the resulting plasmids the produced polyketides were extracted from agar plates and analyzed by HPLC. In extracts from *S. coelicolor* CH999 transformed with plasmid pETR509 or pETR515 no change of the HPLC profile compared to control extracts was observed. *S. coelicolor* CH999/pETR509 produced two major compounds which, according to HPLC and UV/VIS spectrum analysis, are identical to the products made by *S. coelicolor* CH999/pSEK33 (SEK15 and SEK15b).

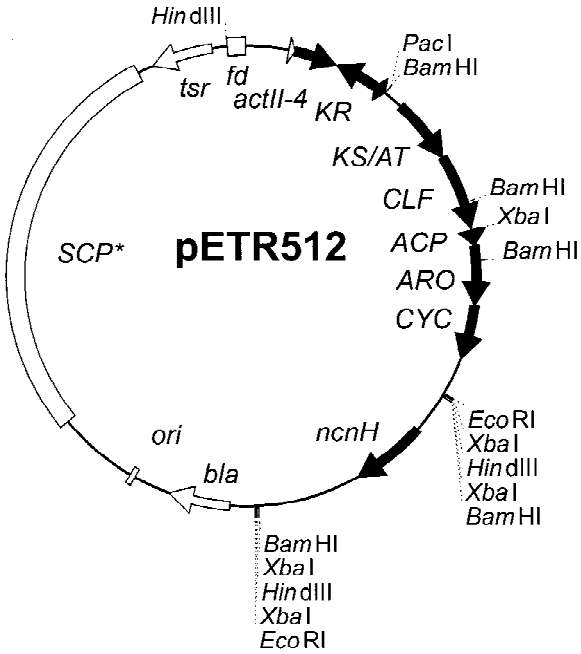

Figure 3. Plasmid map of pETR512 for expression of the naphthocyclinone hydroxylase gene (*ncnH*). The plasmid is a pRM5 derivative in which the 3.5 kb *Bam*HI fragment carrying *ncnH* was cloned as an *Eco*RI fragment downstream of the actinorhodin PKS genes.

Expression of *ncnH* together with the *tcm* PKS and *act* ketoreductase (pETR515) resulted in the production of RM20 and RM20b, c only, compounds which are also made by the *tcm* PKS and *act* ketoreductase alone indicating that either *ncnH* was not expressed as an active enzyme or that the hydroxylase did not accept these core polyketides as a substrate. In contrast, when *ncnH* was coexpressed with the *act* ketoreductase, PKS, aromatase and cyclase on the plasmid pRM5, a new peak was detected on HPLC profiles (Figure 4). The compound represented by this peak, designated P-512, with a different UV/VIS spectrum compared to aloesaponarin II and 3,8 dehydroxy-methyl-anthraquinone-carboxylic acid (DMAC) which are made with pRM5 alone, was isolated, purified and the chemical structure was determined by NMR and MS.

Similarly, there also appeared an additional peak when *ncnH* was expressed in combination with the *act* ketoreductase, *fren* PKS, *fren* aromatase and the *act* cyclase on the plasmid pSEK26 (data not shown). This peak probably represents a novel hydroxylated compound. Purification and structural analysis of this molecule is underway to show if hydroxylation of SEK26 occurred. Since aloesaponarin II and SEK26 are very

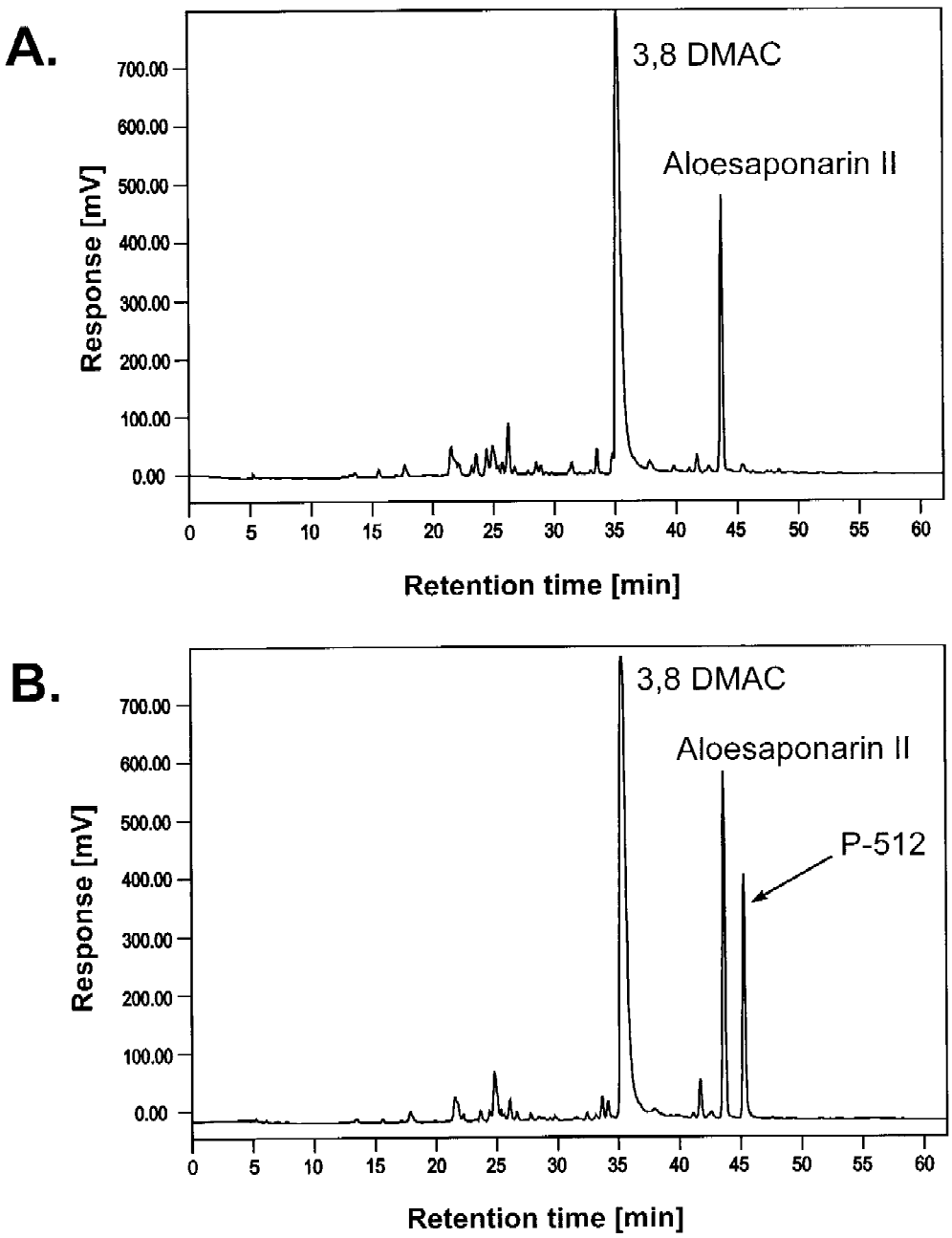

Figure 4. HPLC profiles of extracts from *S. coelicolor* CH999 harboring the plasmids pRM5 (A) and pETR512 (B), respectively. The elution was performed as described in materials and methods using an acetonitrile:H_2O gradient from 20–80% (containing 1% acetic acid) in 45 min. The absorbance at 280 nm was detected.

similar structures it can be suggested that the naphthocyclinone hydroxylase accepts at least those two compounds as a substrate for hydroxylation. A common structural feature of aloesaponarin II and SEK26 is a 5-hydroxy-1,4-naphthoquinone moiety which is not found in SEK15b and RM20. Since aloesaponarin II and SEK26, but not SEK15b and RM20, are substrates for NcnH this 5-hydroxy-1,4-naphthoquinone moiety might be essential for substrate recognition and activity of the naphthocyclinone hydroxylase whereas the size of the molecule seems to play only a minor role.

Structure elucidation and characterization of the compound P-512

1,4,7-trihydroxy-9-methylanthraquinone (hydroxyaloesaponarin, P-512) was purified to homogeneity by preparative HPLC. 1,4,7-Trihydroxy-9-methylanthraquinone was obtained as reddish crystals, mp 250 °C (decomp.). The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz.

^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 19:1), δ (ppm), mult., J (Hz): 13.3, s, 1-OH; 12.7, s, 4-=H; 7.49, d, J_{6-8}=2.7, 6-H; 7.17, d, J_{2-3}=9.3, 2-H; 7.12, d, J_{2-3}=9.3, 3-H; 6.90, d, J_{6-8}=2.7, 8-H; 2.71, s, 9-CH_3. ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 19:1), δ (ppm): 188.1 C-10, 187.4 C-5, 161.7 C-7, 156.6 C-1, 156.6 C-4, 136.9 C-5a, 129.3 C-2, 127.0 C-3, 125.2 C-8, 123.5 C-9a, 113.4 C-10a, 112.8 C-4a, 111.8 C-6, 23.9 9-CH_3. EIMS (70 eV), *m/z* (rel. int.): 270.0533 (100%, m^+, $C_{15}H_{10}O_5$ requires 270.0528), 252 (11%), 241 (5%), 224 (7%), 213 (6%), 139 (6%), 125 (10%).

The molecular weight of 1,4,7-trihydroxy-9-methylanthraquinone is 270, and high resolution mass spectrometry suggested that its composition is $C_{15}H_{10}O_5$. This is in agreement with the NMR data, although two of the carbon signals overlap (C-1 and C-4) while one of the three hydroxyl protons was exchanged for deuterium by the solvent and not observed in the ^{1}H NMR spectrum. The unsaturation index of the compound is consequently 11, which fits with an anthraquinone derivative. The ^{1}H couplings observed suggest the presence of two benzene rings, one 1,2,3,4-tetrasubstituted and one 1,2,3,5-tetrasubstituted, and the complete structure could be elucidated based on the ^{1}H-^{13}C correlations observed in the HMQC and HMBC (summarized in Figure 5) spectra. The two hydrogen bonded hydroxyl protons each give long-range correlations to the hydroxylated carbons (C-1 and C-4) as well as to the two adjacent carbons (C-2/C-10a and C-3/C-4a). This closes the first benzene ring, and long-range correlations between 2-H and C-10 as well as between 3-H and C-5 supports the idea of an anthraquinone. The second benzene ring is substituted by a methyl and a hydroxyl group, and long-range correlations between the methyl protons and C-8, C-9, C-9a and C-10, as well as between 6-H and C-5, C-8 and C-9a determine the structure. Although the structure is not really a new one – it has previously been reported as a synthetic intermediate in the chemical synthesis of tetracenomycins (Cameron & de Bruyn 1992) and furthermore the same metabolite has been described as 5-hydroxy-aloesaponarin II in a mutant of *S. coelicolor* (B22) (Bystrykh et al. 1997) – the isolated compound is a novel recombinant hybrid polyketide.

When purified P-512 was tested in activity assays with *E. coli* and *B. subtilis* as target organisms a significant activity against *B. subtilis* was observed in the range from 100 to 12.5 μg/ml pure compound, indicated by a 50% reduction of cell density (OD_{600}) and a 20% increase in SYTOX Green fluorescence. No activity against *E.* coli was detected. Unfortunately, P-512 is also cytotoxic in a concentration of 0.7 μg/ml which is not surprising due to the quinone structure of P-512 which can lead to the formation of radicals under anaerobic conditions.

Conclusions

From the structure of naphthocyclinone several potentially interesting tailoring enzymes (e.g. a hydroxylase, an O-methyltransferase and an O-acyltransferase) are expected to be involved in biosynthesis of this aromatic polyketide. A putative hydroxylase gene from the naphthocyclinone cluster (*ncnH*) was functionally expressed in combination with the ketoreductase, min. PKS, aromatase and cyclase of the actinorhodin cluster from *S. coelicolor* leading to the production of a novel, hybrid hydroxylated polyketide molecule demonstrating that *ncnH* indeed encodes an active hydroxylase. Gene replacement of *ncnH* in the chromosome of *S. arenae* abolished naphthocyclinone production in the resulting mutant strain (data not shown), demonstrating that this hydroxylase is involved in biosynthesis of naphthocyclinone. Additional preliminary results indicate that NcnH also is active on other core polyketides (data not shown). In addition, the newly produced metabolite exhibited significant antibiotic activity against *B. subtilis*. These

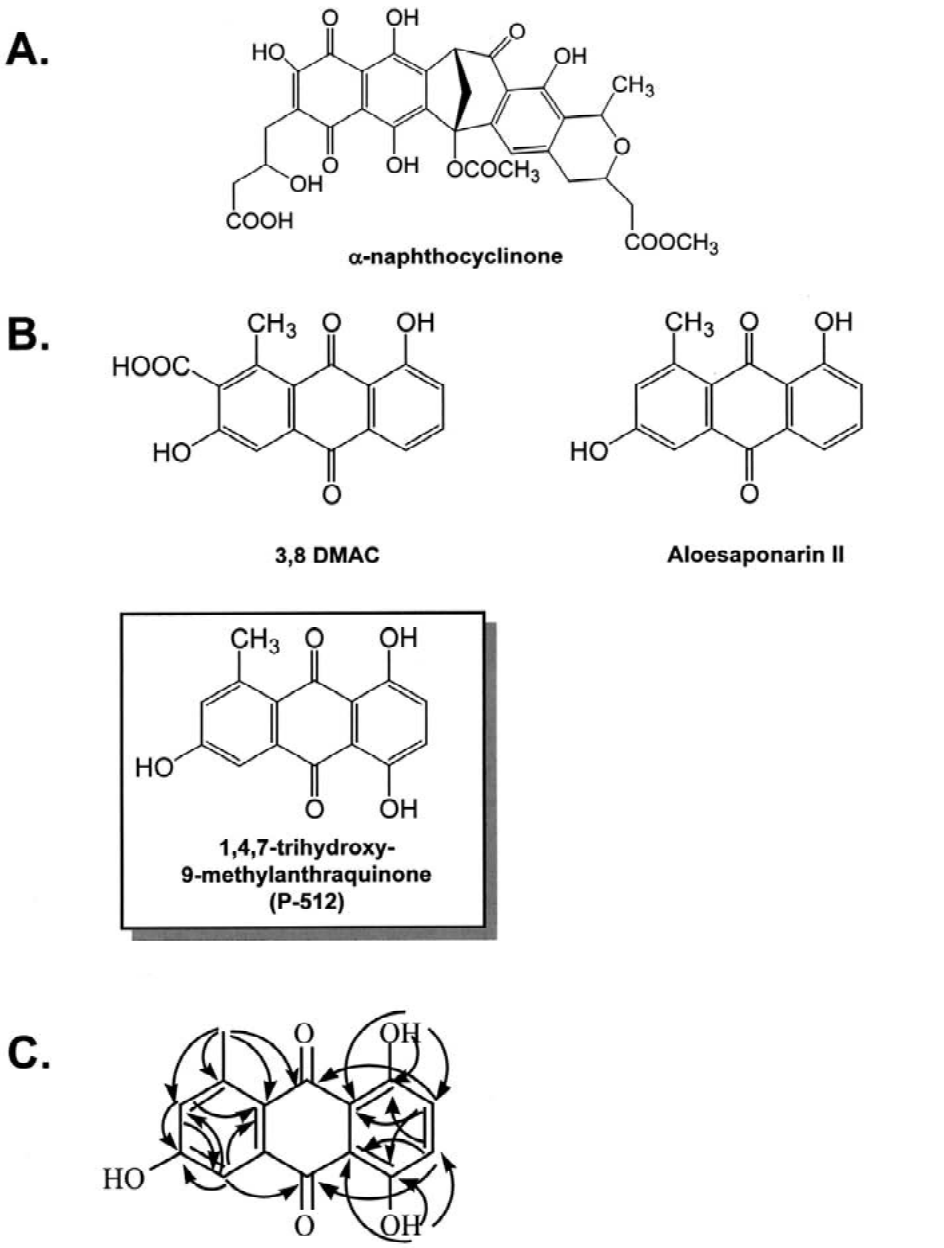

Figure 5. Chemical structures of α-naphthocyclinone (A), the metabolites produced by *S. coelicolor* CH999 transformed with the plasmids pRM5 or pETR512 (B) and pertinent HMBC correlations observed with 1,4,7-trihydroxy-9-methyl-anthraquinone (C).

results demonstrate that the hydroxylase of the *S. arenae* naphthocyclinone gene cluster is a potentially valuable enzyme for the synthesis of 'non-natural natural' polyketides.

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References

Blanco G, Pereda A, Brian P, Mendez C, Chater KF & Salas JA (1993) A hydroxylase-like gene product contributes to synthesis of a polyketide spore pigment in *Streptomyces halstedii*. J. Bacteriol. 175: 8043–8048

Boyer HW & Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41: 459–472

Brünker P, McKinney K, Sterner O, Minas W & Bailey JE (1999) Isolation and characterization of the naphthocyclinone gene cluster from *Streptomyces arenae* DSM40737 and heterologous expression of the polyketide synthase genes. Gene 227: 125–135

Bullock WO, Fernandez JM & Short JM (1987) A high efficiency plasmid transforming *recA Escherichia coli* strain with β-galactosidase selection. Bio/Techniques 5: 376–377

Bystrykh LV, Herrema JK, Kruizinga W & Kellogg RM (1997) 5-Hydroxyaloesaponarin II, a minor blue pigment in an actinorhodin-negative mutant of *Streptomyces coelicolor* A3(2). Biotechnol. Appl. Biochem. 26: 195–201

Caballero JL, Martinez E, Malpartida F & Hopwood DA (1991) Organization and functions of the *act*VA region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor*. Mol. Gen. Genet. 230: 401–412

Cameron DW & de Bruyn PJ (1992) Synthesis of tetracenomycins. Tetrahedon Letters 33(38): 5593–5596

Dairi T, Nakano T, Aisaka K, Katsumata R & Hasegawa M (1995) Cloning and nucleotide sequence of the gene responsible for chlorination of tetracycline. Biosci. Biotechnol. Biochem. 59(6): 1099–1106

Decker H, Motamedi H & Hutchinson CR (1993) Nucleotide sequence and heterologous expression of *tcmG* and *tcmP*, biosynthetic genes for tetracenomycin C in *Streptomyces glaucescens*. J. Bacteriol. 175(12): 3876–3886

Fischer J, Maier H, Viell P & Altenbuchner J (1996) The use of an improved transposon mutagenesis system for DNA sequencing leads to the characterization of a new insertion sequence of *Streptomyces lividans* 66. Gene 180: 81–90

Hopwood DA (1997) Genetic contribution to understanding polyketide synthases. Chem. Rev. 97: 2465–2497

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Schrempf H, Smith CP & Ward JM (1985) Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich

Ichinose K, Bedford DJ, Tornus D, Bechthold A, Bibb MJ, Revill WP, Floss HG & Hopwood DA (1998) The granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tü22: sequence analysis and expression in a heterologous host. Chem. Biol. 5: 647–659

Lee S-Y & Rasheed S (1990) A simple procedure for maximum yield of high-quality plasmid DNA. Bio/Techniques 9: 976–979

Lomovskaya N, Otten SL, Doi-Katayama Y, Fonstein L, Liu X, Takatsu T, Inventi-Solari A, Filippini S, Torti F, Colombo AL & Hutchinson CR (1999) Doxorubicin overproduction in *Streptomyces peucetius*: Cloning and characterization of the *dnrU* ketoreductase and *dnrV* genes and the *doxA* cytochrome P-450 hydroxylase gene. J. Bacteriol. 181: 305–318

MacNeil DJ, Gewain KM, Ruby CL, Dezeny G, Gibbons PH & MacNeil T (1992) Analysis of *Streptomyces avermtilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene 111: 61–68

Mandel M & Higa A (1979) Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159

Marsh JL, Erfle M & Weykes EJ (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32: 481–485

McDaniel R, Ebert-Khosla S, Fu H, Hopwood DA & Khosla C (1994) Engineered biosynthesis of novel polyketides: Influence of a downstream enzyme on the catalytic specificity of a minimal aromatic polyketide synthase. Proc. Natl. Acad. Sci. USA 91: 11542–11546

McDaniel R, Ebert-Khosla S, Hopwood DA & Khosla C (1993) Engineered biosynthesis of novel polyketides. Science 262: 1546–1550

McDaniel R, Ebert-Khosla S, Hopwood DA & Khosla C (1995) Rational design of aromatic polyketide natural products by recombinant assembly of enzymatic subunits. Nature 375: 549–554

Oki T (1988) in JW Lown (Ed.) Anthracycline and anthracenedione-based anticancer agents. Elsevier, Amsterdam: 108

Pridham TG, Hesseltine CW & Benedict RG (1958) A guide for the classification of Streptomycetes according to selected groups. Appl. Microbiol. 6: 52–79

Roth BL, Poot M, Yue ST & Millard PJ (1997) Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. Appl. Environ. Microbiol. 63: 2421–2431

Saitou N & Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425

Sambrook J, Fritsch EF & Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbour Laboratory Press

Sanger F, Nicklen S & Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463–5467

Stassi D, Donadio S, Staver MJ & Katz L (1993) Identification of a *Saccharopolyspora erythraea* gene required for the final hydroxylation step in erythromycin biosynthesis J. Bacteriol. 175: 182–189

Weber JM, Leung JO, Swanson SJ, Idler KB & McAlpine JB (1991) An erythromycin derivative produced by targeted gene disruption in *Saccharopolyspora erythraea*. Science 252: 114–116

Wierenga RK, Terpstra P & Hol WGJ (1985) Interaction of pyrophosphate moieties with α-helices in dinucleotide binding proteins. J. Mol. Biol. 24: 1346–1357